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Perfluoro-n-decanoic acid (PFDA) effects on protein expression in the rat liver were studied in rodents following in vivo exposure to PFDA levels above, below and at the LD-50. Two-dimensional whole-liver homogenate protein patterns were generated and compared to previous results. As before, numerous proteins were altered; some suppressed, some induced, but most were unaffected. In an effort to identify the altered proteins, further analysis of basic proteins by first-dimension NEPHGE revealed the induction of cytochrome P452 (lauric acid ω -oxidase) and enoyl-CoA hydratase. Induction of these and related enzymes confirms previously observed PFDA-induced peroxisome proliferation and lends strong support to the notion that PFDA blocks normal β -oxidation, causes fatty acid accumulation, and results in compensatory peroxisomal and mitochondrial ω - and β -oxidation. Continued identification of other altered proteins will be undertaken to add to the metabolic paths affected by PFDA to further delineate its toxic mechanism.

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THE MOLECULAR ANATOMY OF PFDA HEPATOTOXICITY AS STUDIED BY TWO-DIMENSIONAL ELECTROPHORESIS

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INTRODUCTION

Perfluoro-n-decanoic acid (PFDA) is a ten carbon straight-chain perfluorinated carboxylic acid whose surfactant properties give it and chemically similar compounds important commercial application. Substantial previous investigation of PFDA's hepatotoxicity has demonstrated that a single 50mg/kg i.p. dose (1) induces significant weight loss characterized by hypophagia (3), hepatomegaly, peroxisome proliferation (4-6), and delayed lethality in rats. More recent observations have led to the notion that PFDA interferes with normal β -oxidation of fatty acids resulting in an accumulation of fatty acids in the cell (2). As a result of this accumulation, peroxisomes proliferate as a prominent feature of compensatory β - (7,8) and ω -oxidative inductions (9,10). The present project, whose progress is described in this annual report, was designed to address the analysis of PFDA hepatotoxicity by examining the protein patterns of rat liver whole homogenates and cell fractions generated by two-dimensional electrophoresis. The overall intent was to evaluate the utility of this approach in toxicity testing by generating 2D liver protein patterns representing various levels of PFDA intoxication with the assumption that changes in hepatocellular protein expression can serve as specific indicators of cell injury and dysfunction.

First year objectives, whose results were previously reported [and published (12)], involved the integration of the large scale ISO-DALT system into our previously small-scale approach (i.e. 20 2D gels per run versus 6-12 gels per run). Results obtained from pilot experiments (funded by AFGSR-SFRP) were reproduced and confirmed. Second year objectives, abbreviated from those presented in the original proposal, are restated as follows:

In the ensuing two years of the project it is our aim to identify conclusively the polypeptides altered by various levels of PFDA intoxication in each liver cell fraction and to have statistical data derived from image analysis concerning variations in specific spot intensity and x,y coordinate position (charge shift). Sharing our imaging data with other laboratories conducting toxicologic studies of liver will provide us with access to reference markers for those proteins we may have difficulty identifying. Consequently, the identification of such proteins in the pattern will promote the continued development of a protein database for this laboratory and others.

Regarding these objectives, our efforts during the second year of the project focused on the identification of proteins known to be involved in hepatic lipid metabolism and suggested by other AFOSR supported research to be altered by PFDA exposure. The results of these endeavors (some categorical; others strong estimates) are described in this report.

Preliminary image analysis of 2D gel patterns, in conjunction with scientists at Eli Lilly and Co., though planned, was not carried out. Due to personnel changes at Lilly accompanied

by altered policy and unexpected logistical difficulties, this planned collaboration was not fruitful. However, during the latter stages of year two, additional funds from AFOSR enabled us to acquire the KEPLER 2-D Gel Analysis System and data recently obtained using the system has been included in this report. The KEPLER System enables us to digitize the >1000 protein spots that appear on a single liver whole homogenate 2D gel and compare samples from large experiments (n=20-100) giving statistical validity to our conclusions. Perhaps more importantly, this imaging process enables us to identify protein pattern variations [i.e. proteins whose expression varies (induction/decline after toxicant exposure) as a group] as well as proteins correlated [co-regulated] or anti-correlated [anti-coregulated] relative to exposure level.

Despite their proven utility, conventional 2D techniques used in our experiments have inherent difficulty in generating isoelectric focusing gradients at extremes of pH with broad range ampholytes, proving especially unstable at alkaline pH. Consequently, proteins with alkaline pI's were consistently poorly resolved. Our approach to visualizing basic proteins has been to run NEPHGE [19] gels in the first dimension followed by conventional SDS-PAGE in the second. Due to the acidic pH required of the NEPHGE sample buffer, protein solubility is compromised and therefore some proteins fail to enter the gel while others fail to form discrete spots, resulting in horizontal streaks. To identify mitochondrial proteins whose expression is altered by PFDA intoxication, we exploited the NEPHGE-DALT approach. However, due to problems mentioned above, basic proteins of particular interest to our studies were poorly resolved. The use of alkyl maltosides to improve mitochondrial cytochrome c oxidase purification without loss of electron transfer ability [20] and the electrophoretic mobility in SDS-PAGE of neutrophil-specific membrane proteins [21] prompted us to examine its value in our investigation. In light of these most recent investigations, we attempted to improve the resolution of basic mitochondrial membrane proteins by incorporating the alkyl maltoside detergent dodecyl- β -D-maltoside in sample preparation methodology.

MATERIALS AND METHODS

Animal Care and Intoxication. Male Fisher-344 rats were obtained from Charles river Breeding Labs, individually housed, and maintained on rat chow and water *ad libitum*. Rats were injected with a single intraperitoneal dose of 20mg or 50mg PFDA/kg body weight; 100mg PFOA/kg (eight carbon analog); or 3 successive daily doses of 400mg clofibrate/kg. Matched control rats were vehicle injected and pair fed. After 8 days of exposure, rats were sacrificed by halothane anesthesia and livers removed.

Sample Preparation. Liver samples were removed from similar lobes in each rat; one minced and homogenized in 4 volumes of a lysis buffer containing 9M urea, 4% NP-40, 2% β -mercaptoethanol, and 2% ampholytes (Serva pH 9-11) pH 9.5 for ISO-DALT electrophoresis (13). Microsomal and mitochondrial fractions were prepared from a second sample homogenized in sucrose by differential centrifugation (18). These fractions were then solubilized in either a lysis buffer containing 9M urea, 4% dodecyl-maltoside detergent, [and later 2% NP-40 and 2% dodecyl-maltoside detergents], 2% β -mercaptoethanol, 2% ampholyte (Serva pH 2-11) at pH 3.0 (13) for nonequilibrium pH-gradient electrophoresis (NEPHGE-DALT) or the lysis buffer described above for conventional ISO-DALT runs. After complete solubilization, samples were centrifuged at 110,000 x g using a Beckman TL-100 ultracentrifuge and stored at -70°C.

Two-dimensional Electrophoresis. Using the Anderson ISO-DALT System (13), 30 μ L of solubilized protein was placed on each of 20 first dimension gels (25cm x 1.5mm) containing 4% acrylamide, 2% NP-40 (1% NP-40 and 1% dodecyl maltoside, NEPHGE), 2% ampholyte (ISO: BDH pH 4-8; NEPHGE: LKB pH 3.5-10) and electrophoresed (ISO: 27,000 Vhr; NEPHGE: 4,000 Vhr). For conventional ISO-DALT runs, 200 μ g protein in 20 μ L was applied to the first dimension tube gel while 400 μ g (40 μ L) was added for NEPHGE runs. This additional loading was necessitated by the precipitation of the protein sample applied to the top of the NEPHGE gel and its subsequent failure to completely enter the gel for proper separation. Each first dimension gel was then placed on a second-dimension DALT slab gel (20cm x 25cm x 1.5mm) containing a linear 9-17% acrylamide gradient along with molecular weight standards on the gel margin. Internal charge standardization of was accomplished using carbamylated charge-train standards creatine kinase (ISO) and glyceraldehyde phosphate dehydrogenase (NEPHGE). DALT gels were run for 18hr at 4°C and later stained with Coomassie brilliant blue G-250 (14). Protein patterns on some gels were electroblotted onto nitrocellulose for immunological identification of specific proteins. All stained gels and protein blots were photographed on a fluorescent light box with Kodak Panatomic-X film and printed on Ilford Multigrade III photographic paper.

Image Analysis. Stained gels were digitized at 134 micron resolution using an Eikonix 1412 CCD scanner. The gel images were processed using the KEPLER® software system to yield a spotlist giving x,y position, shape, and density information for each detected spot. Furthermore, based on comparisons to master patterns to be developed in our laboratory, such protein spot information will be used to determine statistically significant differences between control and treatment groups (using Student's t-test procedures) as well as correlative studies of protein expression effects (using Pierson product-moment correlation procedures).

RESULTS

NEPHGE-DALT Electrophoresis. Figure 1 illustrates the rat liver mitochondrial fraction NEPHGE-DALT patterns obtained using the two different detergents. Clearly, dodecyl maltoside solubilized proteins formed more discreet spots and migrated farther under the nonequilibrium conditions when compared to those solubilized with NP-40. Subsequent experiments (not shown) demonstrated that a 50/50 mixture of the two detergents resolved the proteins more reproducibly than dodecyl maltoside alone. Consequently, all NEPHGE first-dimension runs now incorporate this nonionic detergent.

PFDA exposure described above was associated with significant alterations of the 2D protein pattern in both mitochondrial (Figure 2) and microsomal (Figure 3) cell fractions tested. PFOA's effect on pattern alteration was similar to PFDA's but less intense. Clofibrate also showed some similarities although compartmental differences in its effect were notable. Foremost among the alterations observed were those illustrated in Figure 2 (panels I-VI). In NEPHGE-DALT separation of the mitochondrial fraction, several proteins with alkaline pI's were induced relative to dose, xenobiotic, and cellular compartment. Catalase (diamond-shaped box) was identified by co-migration of the purified form. With the exception of the thiolase, the following were identified immunologically on protein blots of replicate gels: (A) peroxisomal/microsomal enoyl-CoA hydratase (bifunctional enzyme); (B) cytochrome P451A1

[cyt P452] (lauric acid ω -oxidase); (C) peroxisomal enoyl-CoA hydratase polypeptide I; (D) peroxisomal enoyl-CoA hydratase polypeptide II; (E) 3-ketoacyl-CoA thiolase (tentatively identified by literature comparison (23); and (F) mitochondrial enoyl-CoA hydratase (crotonase) represent the most notable inductions. Panels I-IV represent replicate sample gels from the following treatments: (I) control; (II) 50mg/kg PFDA; (III) 100mg/kg PFOA; and (IV)

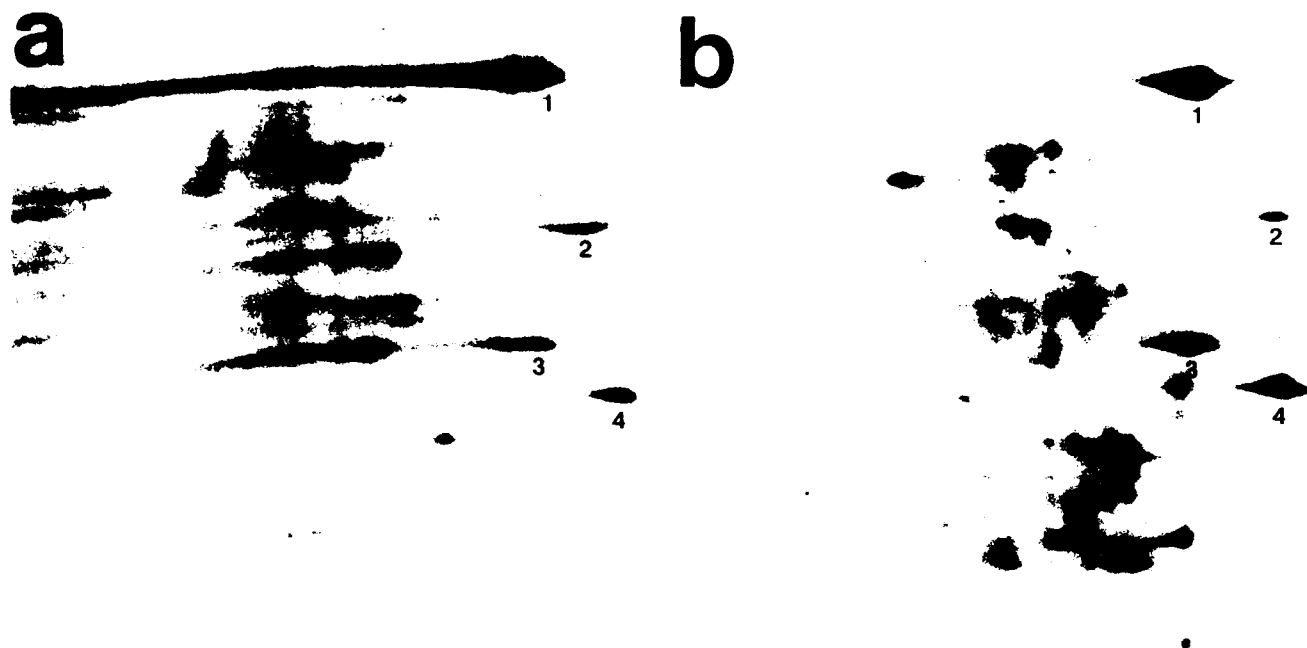


Figure 3. 2-D PAGE (N1 PHGE)-DALT of liver mitochondrial fraction from a rat treated with a peroxisome inducer, perfluoro-*n*-decanoic acid. 400 μ g of protein was loaded and gels stained with Coomassie Brilliant Blue G-250. The 2-D pattern in (A) was generated from proteins solubilized in NP-40 detergent while panel (B) illustrates dodecyl maltoside solubilization. Proteins labeled 1, 3, and 4 are peroxisomal enoyl CoA hydratase (bifunctional enzyme, 78 kDa) and its constituent polypeptides (39.5 and 35.1 kDa). Protein 2 is cytochrome P450IV A1 (lauric acid ω -oxidase, 52.5 kDa).

400mg/kg clofibrate. Panels V and VI are nitrocellulose blots of PFDA-treated patterns exposed to polyclonal antibodies raised against purified rat hepatic bifunctional enzyme (enoyl CoA-hydratase). Panel (V) illustrates the microsomal pattern (corresponds to Fig. 3, panel VIII) (note absence of the mitochondrial protein F, crotonase). Panel (VI) illustrates the mitochondrial pattern (corresponds to Figure 2, panel II).

ISO-DALT Electrophoresis. Microsomal fractions separated by conventional 2D-PAGE are illustrated in Figure 4. Each panel represents replicate gels of several samples from (5) control; (6) 50mg/kg PFDA-exposed; (7) 7 daily 1.25g/kg polyCTFE doses; and (8) 400 mg/kg clofibrate-treated. The encircled charge train resembles the 80 kDa hepatic protein previously shown to be trifluoroacetylated, as shown by others (22). A minor but readily apparent alteration in the composition of that spot-group is induced by PFDA exposure, but not

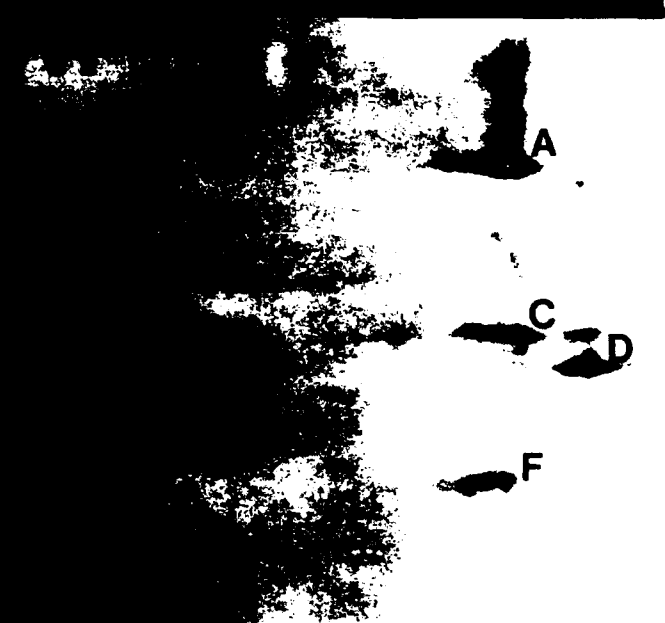
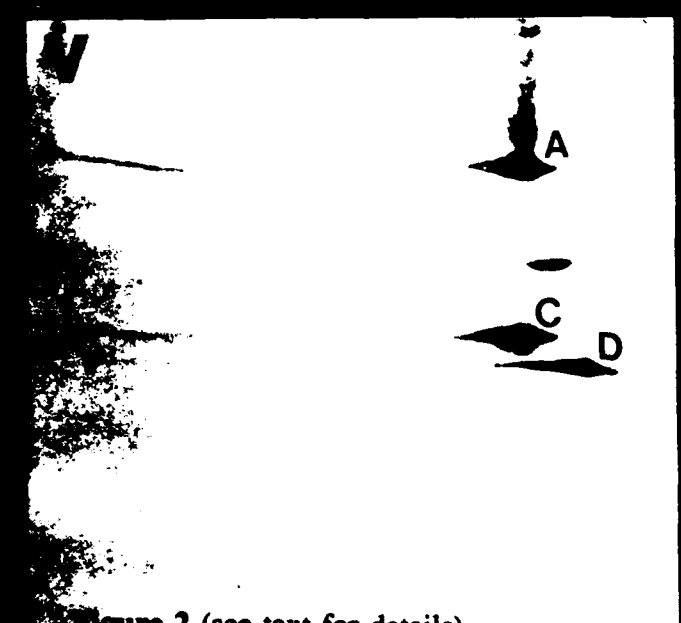
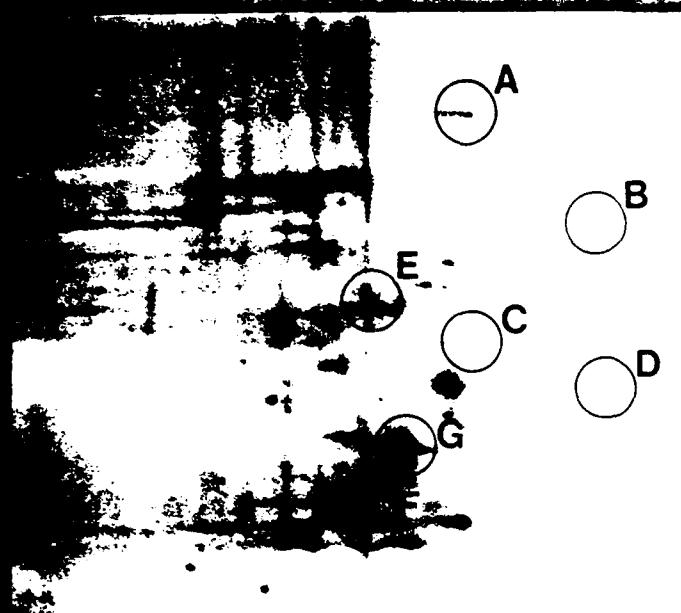


Figure 2 (see text for details)

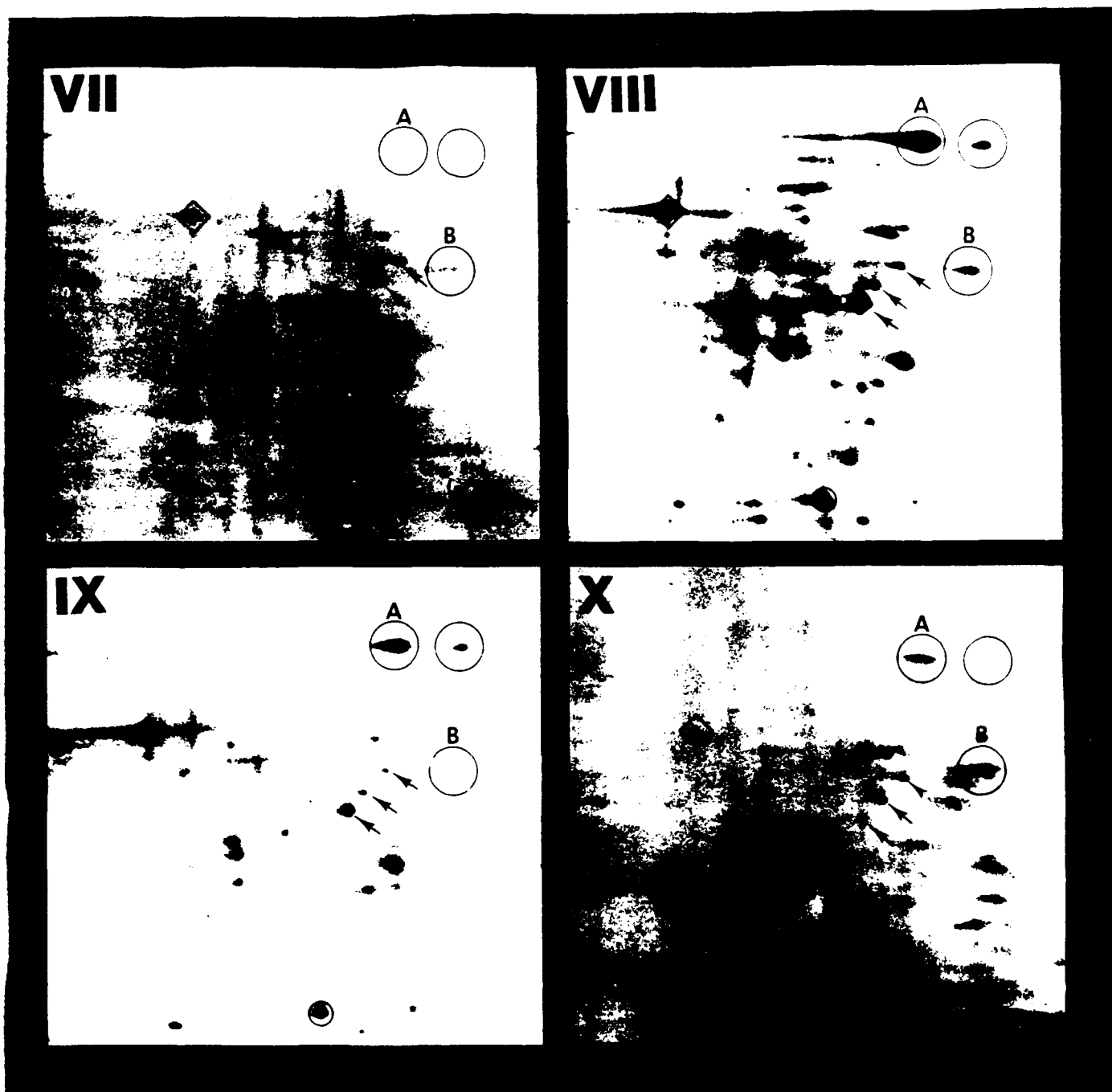


FIGURE 3 Rat liver microsomal protein pattern generated by NEPHGE-DALT. (VII) control; (VIII) 50mg/kg PFDA; (IX) 100mg/kg PFOA; (X) 400mg/kg clofibrate. In this figure and in Fig. 2, protein spot bounded by the diamond is present suggesting that different-sized peroxisomes cosediment with these compounds.

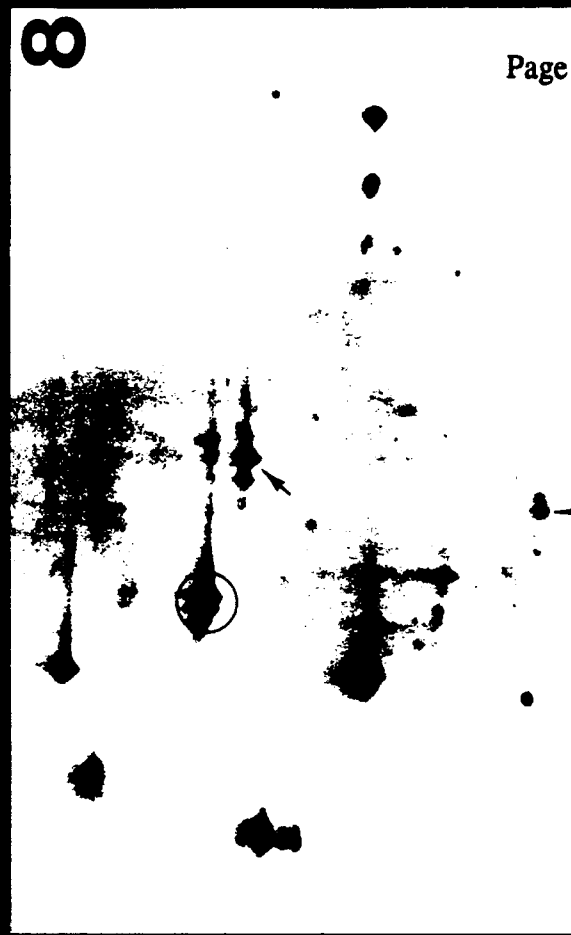
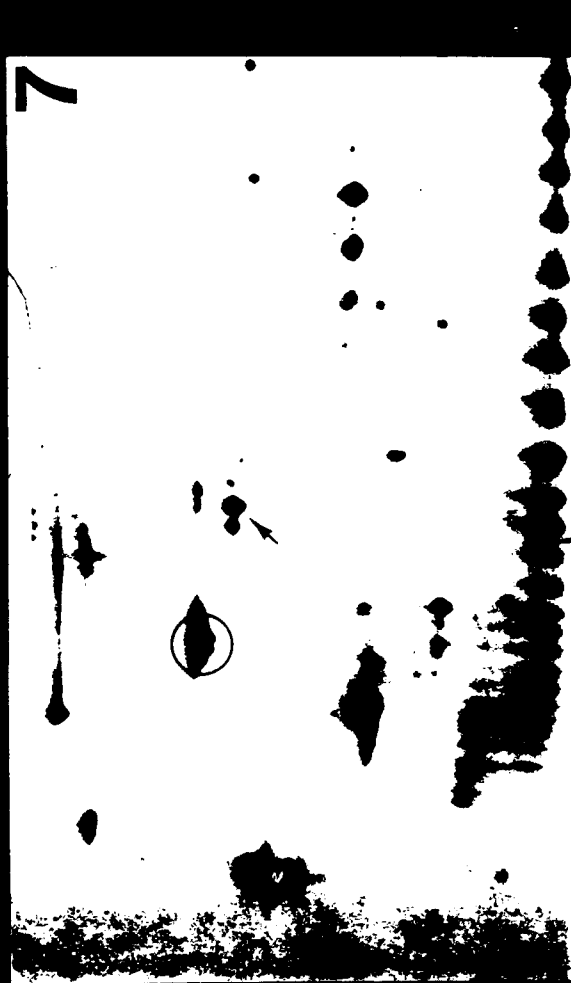
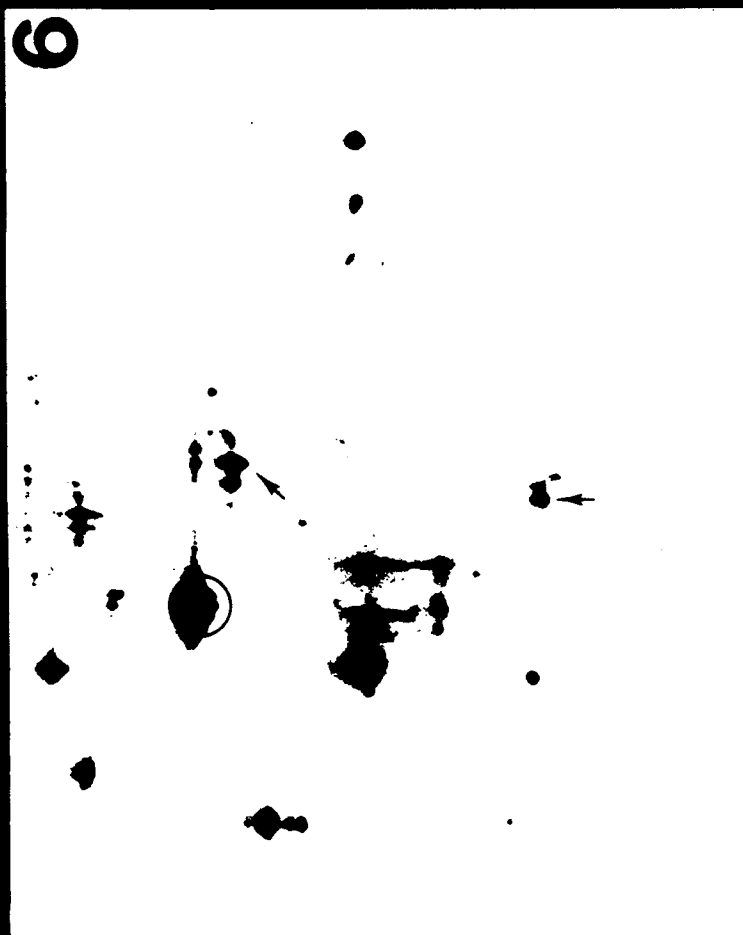
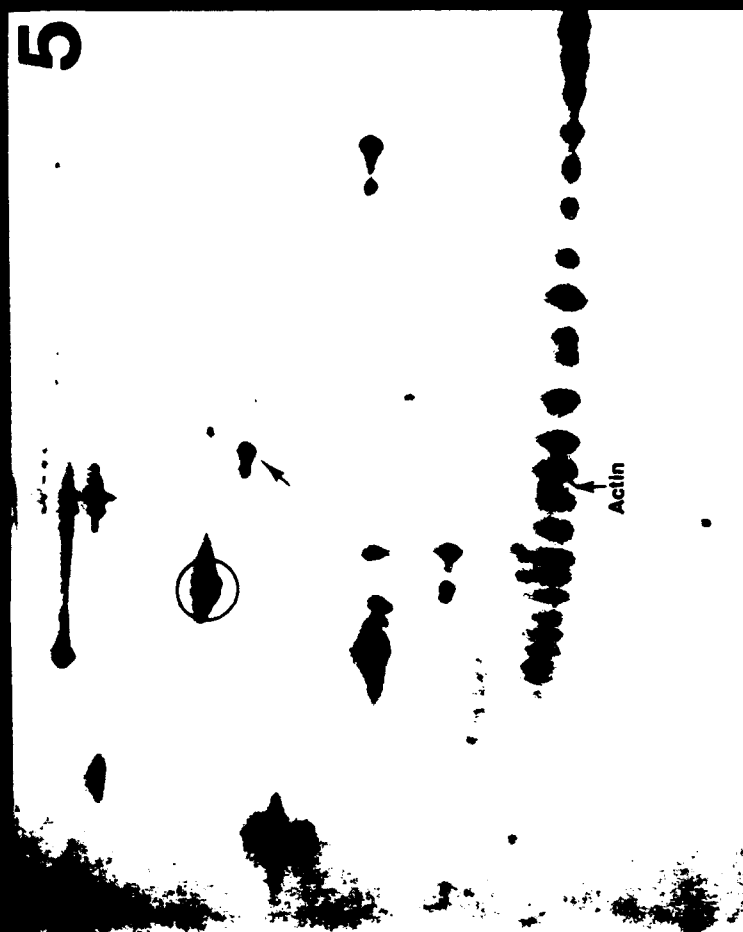


Figure 4 Rat liver microsome protein patterns from conventional ISO-DALT gels. (5) control; (6) 50mg/kg PFDA-exposed; (7) 7 daily 1.25g/kg polyCTFE doses; and (8) 400 mg/kg clofibrate-treated. Creatine kinase charge standard shown in panel 5 and 7.

by polyCTFE nor clofibrate. The nature of this apparent charge shift in the train of proteins suggests protein acylation by PFDA.

At this point in the investigation, as a result of both NEPHGE- and ISO-DALT experiments, we have conclusively identified 17 proteins whose abundance in liver cell fractions has enabled us to observe them reproducibly (Table 1).

TABLE 1. HEPATIC PROTEINS IDENTIFIED BY 2D-PAGE

Protein	MW (kDa)	pI	Method*
actin	43	5.19	<i>l</i>
albumin	66	5.61	<i>l</i>
α_1 antichymotrypsin	59.2	4.29	<i>l</i>
carbamoyl-phosphate synthase	160	5.97	<i>l</i>
catalase	56.4	6.72	<i>c</i>
cytochrome b_5	14.5	4.59	<i>l</i>
enoyl-CoA hydratase (mitochondrial)	26.8	8.01	<i>i</i>
enoyl-CoA hydratase (peroxisomal)	78	8.66	<i>i</i>
enoyl-CoA hydratase subunit I (perox.)	39.5	8.68	<i>i</i>
enoyl-CoA hydratase subunit II (perox.)	35.1	9.23	<i>i</i>
F1 ATPase, β subunit (mitochondrial)	50.6	4.80	<i>l</i>
fatty acid binding protein (FABP)	13.6	6.42	<i>i</i>
3-ketoacyl-CoA thiolase	44.5	7.88	<i>l</i>
lauric acid ω -oxidase (cyt P450IVA1)	52.5	9.15	<i>i,c</i>
myoglobin	17.2	7.15	<i>l</i>
α -tubulin	54.0	4.91	<i>l</i>
β -tubulin	53.8	4.75	<i>l</i>

* estimated pI and MW determined and protein identified by *l* = from the literature; *c* = comigration of purified forms; or *i* = 2D gel immunoblotting.

Image Analysis. The virtues of computerized image analysis with reference to 2D-PAGE and specifically applied to hepatotoxicology studied by 2D-PAGE is reviewed by Anderson (23). Very recent preliminary results gained by using this powerful tool are presented in Figures 5-7. Within the framework of our proposal, we have initially scanned liver whole homogenate gels, produced spot files, and plotted the resulting patterns in these figures. Figure 5 illustrates the processed protein pattern of Fischer 344 whole liver homogenate. Each circle/ellipse represents the information processed and stored for each of over 1300 individual proteins. Each gel pattern becomes a list of spots characterized as to position (x and y coordinates), shape (dx and dy), and amplitude (A). From these values the integrated density (spot volume) can be calculated followed by sound statistical comparisons between corresponding spots from different sample groups (different gels). Individual spots of particular interest can be selected, highlighted, and acted on by any of several statistical processes in KEPLER as shown in Figure 6. Figure 7 demonstrates that each protein spot is given a numerical designation referring to its place in both a master list and individual spot list.

Figure 5 Location of numbered and matched protein spots in preliminary rat-liver whole homogenate 2D electrophoretic pattern. Each circle or ellipse represents a reproducibly resolved protein.

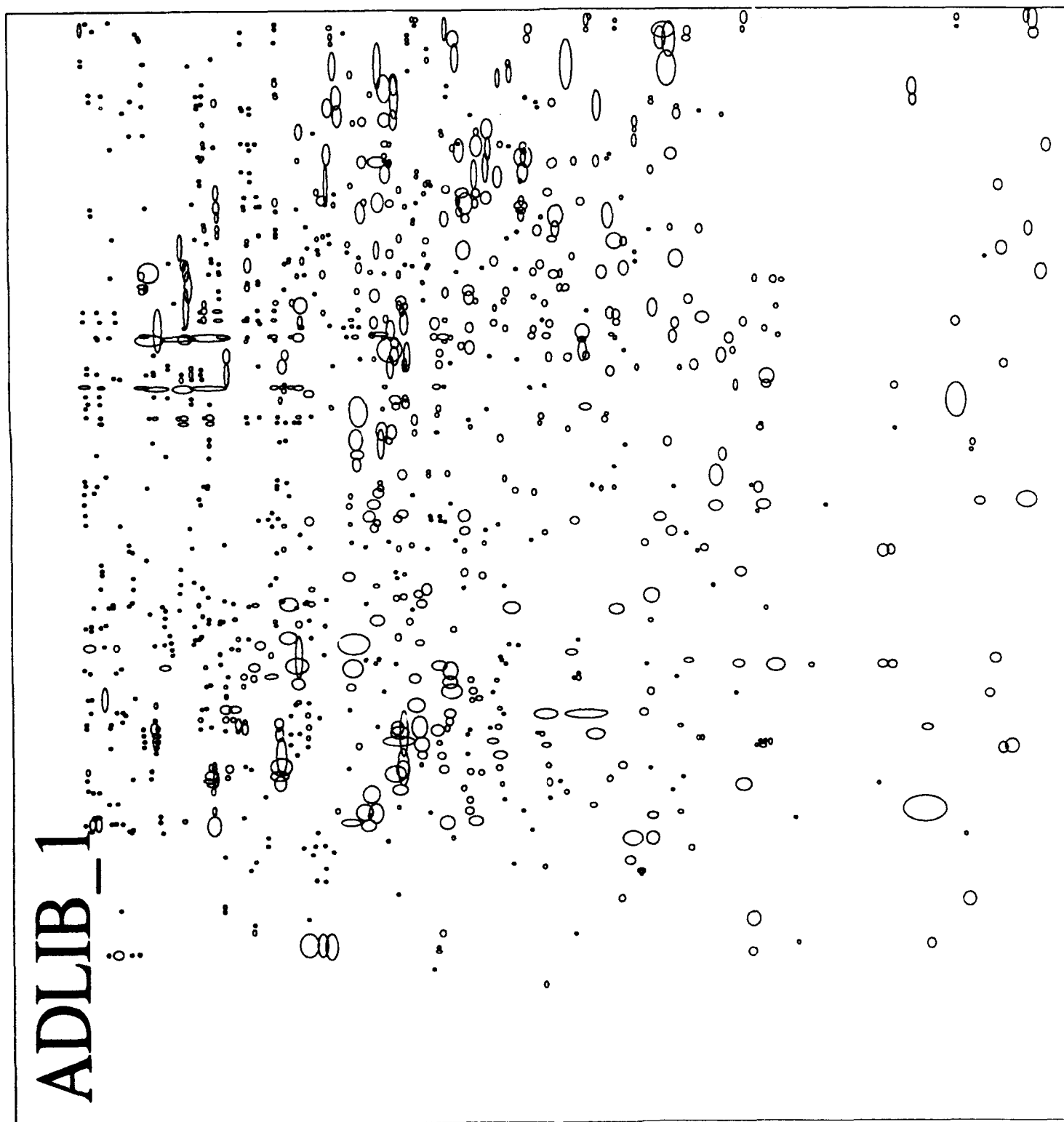


Figure 6 Same as Figure 5, with the exception that specific spots have been arbitrarily selected for emphasis. Such spots can later be used for statistical analysis across 2-100 gel patterns.

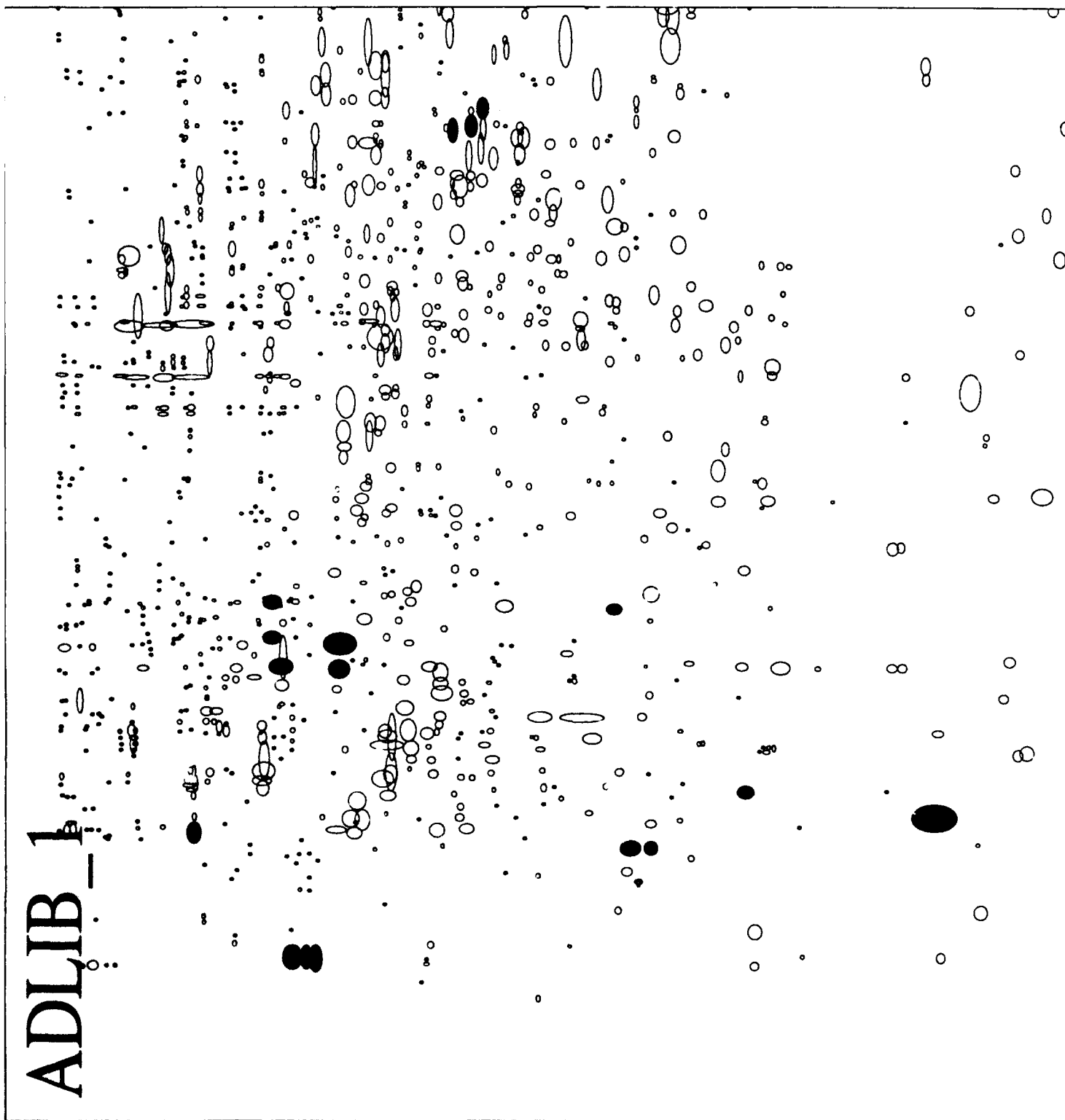
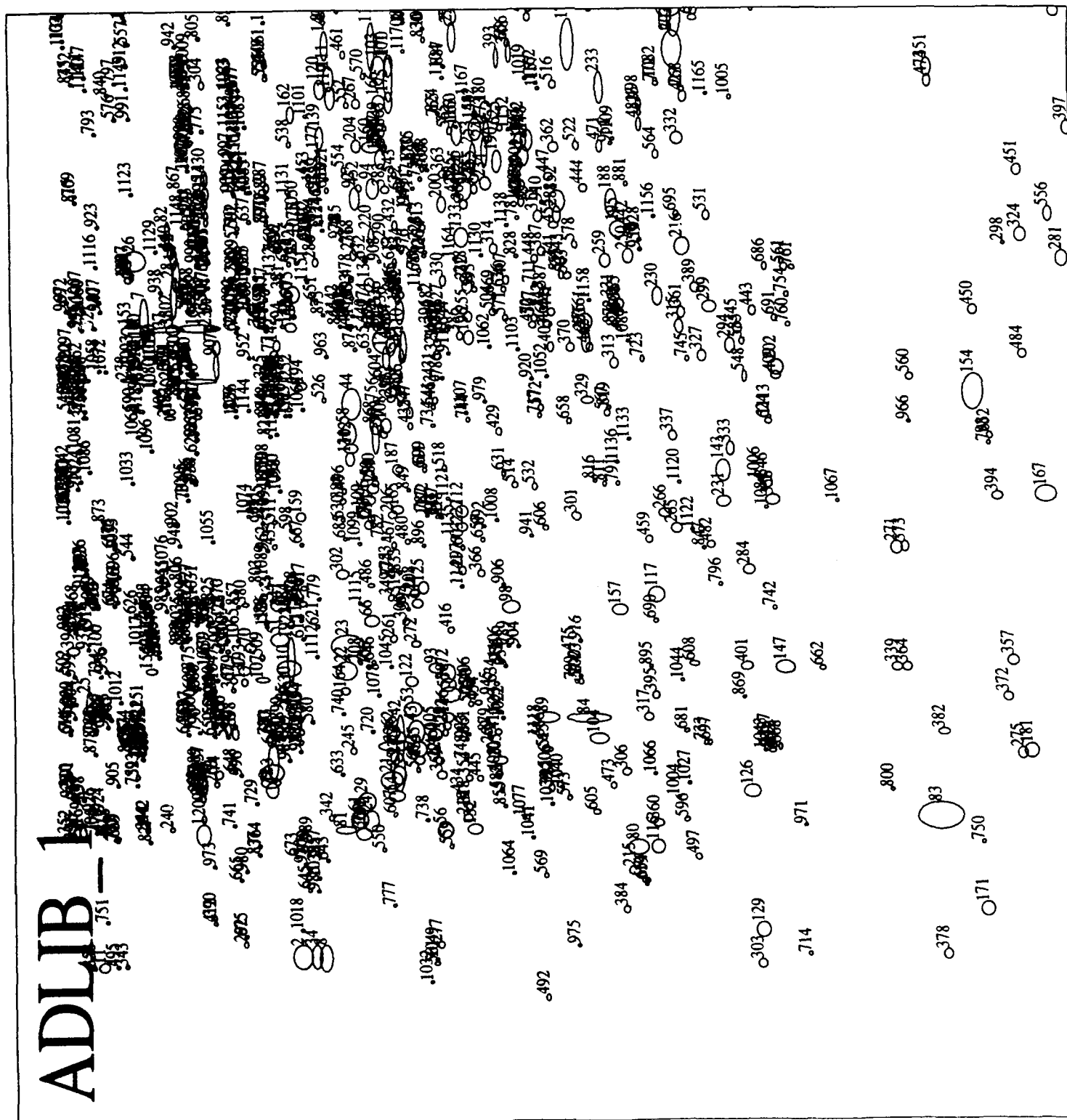


Figure 7

Same as Figure 5, identifying each protein spot with its corresponding spot number. These numbers also identify each protein in the master spot file that contains position, shape, and density information.



DISCUSSION

The cumulative results of this investigation confirm and elaborate the results of traditional toxicologic tests regarding PFDA's hepatotoxic potential. Specific analysis of proteins with basic pI's, presumed to be involved in hepatic lipid metabolism but previously uncharacterized as to 2D-PAGE mobility has yielded important information regarding PFDA's compartmental effects and resulted in a new technical development, *i.e.*, the use of alkyl maltosides to enhance protein resolution. It is clear that PFDA induces significant induction of peroxisomal β -oxidation and to a lesser extent mitochondrial β -oxidation implied by the greatly increased abundance of enoyl-CoA hydratase and 3-ketoacyl-CoA-thiolase (and the minor increase in crotonase) in the mitochondrial/peroxisomal fraction. Furthermore, the confirmed induction of P450IVA1 (lauric acid ω -oxidase) indicates the induction of a second lipid metabolic path by PFDA. These observations persuade one to conclude that PFDA does indeed interfere with normal fatty acid oxidative mechanisms resulting in what appear to be compensatory protein inductions. Consistent with other studies is the lack of induction of fatty acid binding protein by PFDA. Identified immunologically on whole homogenate ISO-DALT gels (not shown), this protein was unaffected by PFDA or PFOA.

The unconfirmed acylation of a hepatic microsomal protein by PFDA suggests that PFDA's hepatotoxic effects are not limited to interfering with lipid metabolism. As an outgrowth of this observation, we are presently investigating protein conjugation by PFDA using mass spectrometry and HPLC. The number and variety of protein expression alterations already observed in our investigation, suggests that PFDA's complicated toxicology involves a number of cell processes representing primary, secondary, or even tertiary toxic effects.

Our rapidly developing capability to image-analyze ISO-DALT gels and to critically evaluate the protein patterns for pattern-alterations puts us in a position to accelerate **protein identification, pattern recognition, and database construction** relative to the elucidation of PFDA's toxic mechanism. During the next year of this investigation, 2D-gel imaging will form our experimental foundation, as we move toward the development of a predictive toxicology based on protein-pattern alterations induced by a variety of agents (in addition to the model compound PFDA) relevant to the Air Force Toxicology program.

Publications resulting from this effort:

Hepatic protein alterations following perfluorodecanoic acid exposure in rats. Witzmann, F.A. and Parker, D.N. **Toxicology Letters** 57:29-36, 1991.

Dodecyl maltoside detergent improves the resolution of hepatic membrane proteins on two-dimensional gels. Witzmann, F.A., Jarnot, B.J., and Parker, D.N. **Electrophoresis** 12:687-688, 1991.

Hepatotoxicity of perfluorinated compounds in male rats: 2D-PAGE analysis. Witzmann, F.A. and Parker, D.N. In: **2-D PAGE '91: Proceedings of the International Meeting on Two-Dimensional Electrophoresis**, M.J. Dunn, Ed. 1991, Zebra Printing, Middlesex UK, pp. 236-237.

Papers presented at scientific meetings:

Induction of an 80kDa protein in rat liver homogenates and cell fractions by perfluoro-n-decanoic acid. Witzmann, F.A. and Parker, D.N. Presented at the 30th Annual Meeting of the Society of Toxicology, February 25-March 1, 1991 in Dallas TX.

Hepatotoxicity of perfluorinated compounds in male rats: 2D-PAGE analysis. Witzmann, F.A. and Parker, D.N. Presented at the International 2D-PAGE Conference, July 16-19, 1991 in London England.

Perfluoro-n-decanoic acid toxicity in the rat liver: Two-dimensional electrophoretic analysis. Witzmann, F.A. Presented as an invited selected topics lecture in *Molecular Pathology: Applications to the Study of Xenobiotic Effects in Animal Testing* at the 43rd National Meeting of the American Association for Clinical Chemistry, July 28-August 1, 1991 in Washington DC; published as an abstract in **Clinical Chemistry** 37:1096, 1991.

Perfluorocarboxylic acid intoxication increases β -oxidative enzyme content of hepatic mitochondrial fractions in rodents. Witzmann, F.A., Jarnot, B.M., and Parker, D.N. Accepted for presentation at the 31st Annual Meeting of the Society of Toxicology, February 23-27, 1992 in Seattle WA.

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